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http://dx.doi.org/10.1289/ehp.1206122

Online 18 January 2013

NIEHS
National Institute of Environmental Health Sciences

National Institutes of Health
U.S. Department of Health and Human Services

The Natural Estrogenic Compound Diarylheptanoid (D3): *In Vitro* Mechanisms of Action and *in Vivo* Uterine Responses via Estrogen Receptor α

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Running title: Diarylheptanoid, ER-dependent action in the uterus

Key words: diarylheptanoid, ER-dependent, nuclear translocation, phytoestrogen, uterus

Acknowledgements: We thank Drs. April Binder and Yin Li for critical review of this manuscript and Drs. Jeff Tucker and Agnes Janoshazi (Fluorescence Microscopy and Imaging Center) Laboratory of Signal Transduction, NIEHS for their assistance in confocal imaging

analysis. Research supports were provided by the Division Intramural Research of the NIEHS to KSK through Z01-ES70065 and LCP through Z01-ES102645, Thailand Research Fund through the Royal Golden Jubilee PhD program (PHD/0255/2546 to PP and WW), the Target Research Grant (DBG5180020) to PP, and the Strategic Basic Research Grant (DBG5280013) to AS.

The authors have no competing financial interests.

Abbreviations:

AF1 (Activation function 1) -

AF2 (Activation function 2) -

D3 (Diarylheptanoid, (3*R*),-1,7-diphenyl-(4*E*,6*E*)-4,6-heptadien-3-ol)) -

E_2 (17β-estradiol) -

EdU (5-ethynyl-2'-deoxyuridine) -

ERE (Estrogen responsive element) -

ERα (Estrogen receptor alpha) -

H2 ERα (D-domain ERα mutant) -

H2 ERα-GFP (D-domain ERα mutant with green fluorescent protein fused) -

ICI (ICI 182,780, pure ER antagonist) -

LBD (Ligand binding domain) -

NLS (Nuclear localization sequences) -

PR (Progesterone receptor) -

αERKO (Estrogen receptor α knockout) -

Abstract

Background: Diarylheptanoid (D3) isolated from the medicinal plant, *Curcuma comosa*, has estrogenic activity.

Objective: To elucidate the mechanisms of D3 actions and compare it to 17β-estradiol (E_2) in both *in vitro* and *in vivo* uterine models.

Methods: We used human uterine (Ishikawa) cells to determine the estrogenic action of D3 on activation and nuclear translocation of estrogen receptor α (ERα). In addition, we further characterized the uterine response to D3 treatment *in vivo*.

Results: D3 activated an estrogen responsive element (ERE)-luciferase reporter through ERα and molecular modeling suggested that D3 could be accommodated in the ERα binding pocket. By using a modified ERα to assay ligand-dependent nuclear translocation, we demonstrated D3-dependent ERα interaction and translocation. In mouse uteri, D3 treatment increased early and late phase estrogen-regulated gene responses in ovariectomized wild type animals, similar to E_2; no response was seen in ERα knockout animals. D3 shows a divergence in estrogen responses by inducing robust DNA synthesis in uterine epithelial cells, linked to an increase in cell-cycle related genes; however, D3 showed no uterine weight increase at 24 h. D3 also affected uterine progesterone receptor expression patterns similar to E_2. When administered together, no additive or antagonistic effects of D3 on E_2 were observed. Our findings suggest that D3 is a weak estrogenic agonist compound.

Conclusion: D3 is a weakly acting phytoestrogen that mimics the mitogenic responses produced by E_2 in an ERα-dependent manner but is unable to increase uterine weight nor enhance or antagonize the effects of estrogen.

Introduction

Estrogens play important roles in growth, differentiation and maintenance functions of many target tissues in the female reproductive organs (Couse and Korach 1999). The biological actions of estrogen are mediated primarily through estrogen receptor (ER) α and β proteins (Couse et al. 1997). ERs are members of the nuclear receptor family of proteins containing multiple functional domains. The A/B domain harbors the activation function 1 (AF1). The DNA binding domain is located in the C region of the receptors. The hinge region (D-domain) contains nuclear localization sequences (NLS) (Mader et al. 1993). The E/F domains contain the ligand binding region and AF2 function. AF1 and AF2 portions of the protein facilitate the transcriptional activity of the ER (Tora et al. 1989). Upon binding ligand, ER is localized to the nucleus and initiates gene transcription through multiple pathways, including classical ERE-dependent, and non-classical pathways (Hall et al. 2001).

The uterus is one of the most prominent estrogenic responsive target tissues, predominantly expressing ERα (Couse et al. 1997). Uterine response to estrogen is rapid and eventually leads to a dramatic increase in cell proliferation (Martin et al. 1973). However, the uterotrophic responses to estrogen vary with time after hormone exposure. An early response of water imbibition in uteri is mediated through ERα as ERα knockout (αERKO) mice show no water imbibition and no increase in uterine weight with E_2 treatment (Korach 1994). The genomic responses of the uterus to E_2 have been reported from 0.5 to 96 h (Hewitt et al. 2003; Naciff et al. 2007). Some exogenous estrogens (bisphenol A and genistein) as well as one of the endogenous estrogens (estriol) are considered weak estrogens in the uterus. Weak estrogenic compounds are less potent than E_2; they exhibit early uterine responses, but are less effective in their abilities to cause robust subsequent uterine responses such as cellular hypertrophy and

hyperplasia (Hewitt and Korach 2011). Stronger estrogens including E_2, initiate both early and late effects (Anderson et al. 1975). Transcripts that increase 1 to 2 h after acute dosing of estrogenic compounds are components of the E_2 responsive 'early gene cluster', including *Fos* and *Inhbb* (Inhibin beta-B) (Hewitt et al. 2003). The late responses include the increased and sustained RNA and protein synthesis, which lead to uterine cellular hypertrophy, DNA synthesis and hyperplasia (Hewitt et al. 2003), as well as an alteration of progesterone receptor (PR) expression patterns (Mote et al. 2006). A second response phase is characterized by a wave of mitosis and DNA synthesis, which occurs 16 to 24 h after E_2 treatment, and is correlated with the late phase cell cycle regulators, including *Aurkb* (aurora kinase B) and *Ccnb2* (cyclin B2) (Hewitt et al. 2003; Hewitt and Korach 2011). The early and late events reflect the uterotrophic action of estrogens on uterine tissues and have been widely used to evaluate and compare potency, estrogenic or antagonistic activity of xenoestrogenic compounds.

Diarylheptanoids are phytoestrogens isolated from *Curcuma comosa,* a plant in the Zingiberaceae family. *C. comosa* has been marketed as a plant-derived dietary supplement product, traditionally used in indigenous medicine as an alternative remedy for hormone replacement therapy in menopausal women (Piyachaturawat et al. 1995). Other diarylheptanoids are found in *Curcuma* as well as other plants in the ginger family (Keserü and Nógrádi 1995). D3 (Figure 1A), one of the most abundant purified diarylheptanoids from *C. comosa* rhizome extract (Suksamrarn et al. 2008), exerts the most potent estrogenic activity when administered for 2 or 3 consecutive days in a rodent uterine bioassay (Winuthayanon et al. 2009a; Winuthayanon et al. 2009b). We previously reported that D3 also contains a vascular relaxative effect in the endothelial cells of rat aortic rings, similar to the effect of estrogen (Intapad et al. 2012). These biological actions of D3 may potentially be a benefit to women without causing adverse side

effects known to be caused by current or traditional estrogen replacement therapy (Shifren and Schiff 2010). Due to the high bioavailability (Suksamrarn et al. 2008), the estrogenic-like bioactivities of D3, and the long term favorable use of these plant products by daily consumption in the form of dried fine rhizome power in capsules as well as decoctions twice a day, we aimed to characterize the *in vitro* and *in vivo* mechanism(s) of action of D3 focused on its effect in uterine cells. The estrogenic activities of D3 on wild type (WT) and ERα mutant receptor in a human uterine (Ishikawa) cell line were evaluated as well as evidence of D3 binding to the ERα using a new cellular assay for detecting direct interaction of D3 to the ERα. In addition, both early and late biological responses in the mouse uterus were also evaluated, both early and late events, including any potential effect on modulating the action of E_2. This work demonstrates that D3 has weak estrogenic activity that is mediated through ERα, and does not synergize or antagonize E_2 effects in both a human uterine cell model and rodent uterus.

Methods

Chemicals. 17β-estradiol (E_2) was purchased from Sigma (St. Louis, MO, USA). ICI 182,780 was purchased from Tocris Bioscience (Ellisville, MS, USA). All chemicals were dissolved in ethanol unless otherwise indicated. D3 ((3*R*)-1,7-diphenyl-(4*E*,6*E*)-4,6-heptadien-3-ol; Figure 1A), was isolated from *C. comosa* as described previously (Suksamrarn et al. 2008).

Three dimensional modeling of D3. The model for D3 was created in Insight II version 2005 (Accelrys Inc., San Diego, CA, USA) and minimized using the Discover_3 force field. The model was manually superimposed onto the structure of trifluoromethylphenylvinyl estradiol (TFMPV-E_2) in the crystal structures of TFMPV-E_2 bound to the ligand binding domain (LBD) of ERα (ERαLBD, PDB ID code 2P15), and also E_2 bound to ERαLBD (PDB ID code 1GWR)

followed by additional minimization of the ligand docked to the crystal structure of P215 to relieve any significant strain that may have been created from the manual modeling.

Plasmids. The expression plasmids for mouse pcDNA3-WT-ERα (wild type (WT) ERα) (Winuthayanon et al. 2009a), and pcDNA3-H2-ERα (D-domain ERα mutant or H2 ERα), the disrupted NLS mutant of ERα, and pcDNA3-H2-ERα-EGFP (D-domain ERα mutant with GFP fused or H2 ERα-GFP) (Burns et al. 2011) were used. The H2 ERα has modification in its nuclear localization sequence such that the H2 ERα remains predominantly localized in the non-nuclear compartment in the absence of ligand and translocates to the nucleus when bound and interacting with the ligand (Burns et al. 2011). The 3×ERE-TATA-Luc-expressing plasmid was a gift from Dr. Donald McDonnell, Duke University Medical Center, Durham, NC, USA. pRL-tk (constitutively expressed renilla) was purchased from Promega (Madison, WI, USA).

Cell culture and transfection conditions. Human endometrial adenocarcinoma, Ishikawa ER-negative cells, which do not express endogenous ER were a gift from Dr. Richard DiAugustine, National Institute of Environmental Health Sciences (NIEHS), NC, USA (Ignar-Trowbridge DM 1993). Human cervical epithelial cell line HeLa was purchased from ATCC (Manassas, VA, USA). Cell-culture reagents were purchased from Invitrogen Life Technologies (Invitrogen, Carlsbad, CA, USA), unless otherwise indicated. Cell culture conditions were described previously (Winuthayanon et al. 2009a), additional information see Supplemental Methods.

Confocal Microscopy. HeLa cells were used for the GFP-tagged H2 ERα translocation experiment due to high transfection efficiency. HeLa cell culture and treatment conditions were previously described (Burns et al. 2011). Briefly, HeLa cells were plated on Lab-Tek 2-well chamber slides (NUNC, Rochester, NY, USA) overnight. Cells then were transfected with 0.4 μg

of H2 ERα-GFP in DMEM supplemented with 10% SFBS for 8 h. At 27 h after the transfection, cells were treated for 3 h with ethanol (as a vehicle or Veh), E_2 (10 nM), or D3 (50 μM). Then, cells were fixed and visualized on a Zeiss 510-UV meta for the cellular localization of H2 ERα-GFP as previously described (Burns et al. 2011). The cellular colocalization of H2 ERα-GFP and DAPI (for nucleus) was quantified with the Multi Wavelength Cell Scoring application from MetaMorph Microscopy Automation and Image Analysis Software (version 7.7.0.0, Molecular Devices).

Uterine bioassay in adult wild type ovariectomized mice. Animals were handled according to NIEHS Animal Care and Use Committee guidelines and in compliance with a NIEHS approved animal protocol. The animals were treated humanely and with regard for alleviation of suffering. Adult female C57BL/6J mice (8-week-old) were purchased from Charles River Laboratories (Raleigh, NC, USA). C57BL/6J αERKO mice (Lubahn et al. 1993) were generated at Taconic Farms (Germantown, NY, USA). All mice were ovariectomized (OVX) and left for 2 weeks to recover and eliminate the endogenous ovarian steroids before the study. Mice were randomly grouped and treated for 2 or 24 h with sesame oil (Veh; subcutaneous administration or s.c.), D3 (100 mg/kg) dissolved in 100 μL sesame oil (s.c.), or E_2 (10 μg/kg) dissolved in 100 μL saline (intraperitoneal administration or i.p.). In some experiments, WT OVX animals were treated with both D3 (100 mg/kg) and E_2 (10 μg/kg). EdU (5-ethynyl-2'-deoxyuridine; 2 mg/mL in 100 μL of phosphate buffered saline) injection was used to measure DNA synthesis but only for the 24 h time point, and was delivered as a second injection, 2 h prior to tissue collection (22 h after Veh, E_2, or D3 injection). Animals were euthanized by using CO_2 asphyxiation. Tissue collection and real-time PCR were performed as described previously (Winuthayanon et al. 2010), additional information see Supplemental Methods.

Statistical analysis. The results were expressed as mean and standard error of means (X ± S.E.M). The statistical difference among different groups were compared using one way analysis of variance (ANOVA) followed by Tukey post test, or two way ANOVA followed by Bonferroni post test. Statistical significance is considered when $p < 0.05$.

Results

Modeling of D3 to ERα supports agonist binding. To model potential D3 binding to ERα, 3D molecular docking was used. The structure of E_2 bound to the ligand binding domain (LBD) of ERα does not indicate how D3 could behave as an agonist. Because D3 is a larger molecule than E_2, there does not appear to be enough space in the ERα binding pocket to accommodate D3 binding. However the crystal structure of the potent ERα agonist trifluoromethylphenylvinyl estradiol (TFMPV-E_2) bound to ERα suggests flexibility and conformational changes in the binding pocket allowing accommodation of the bulkier TFMPV-E_2 ligand (Nettles et al. 2007), specifically the unwinding of helix 7 and the alteration of the side chains M342, M421, and F425 (Figure 1B-C). Superimposing D3 onto TFMPV-E_2 and then minimizing the ERα binding pocket, suggests that ERα could potentially accommodate D3 in a similar agonist type binding mode as when TFMPV-E_2 is bound (Figure 1C).

D3 activates ERα-dependent transcription. Our previous study in liver cancer (HepG2) cell lines and the molecular modeling suggested that D3 could act as an agonist with ERα (Winuthayanon et al. 2009a). We then further evaluated the mechanism of D3 on ERα-mediated transcriptional activity *in vitro* in the uterine cell model. Plasmids containing WT ERα and 3×ERE-Luc were transiently transfected into Ishikawa cells. In the presence of WT ERα, E_2 at a dose of 10 nM significantly ($p < 0.001$) increased the luciferase activity compared to Veh control

and E_2-induced transcription was fully inhibited by ICI 182,780 (ER antagonist, ICI) (Figure 2A). D3 significantly stimulated ERE-dependent luciferase activity in a dose-dependent manner, with the maximum luciferase activity at doses of 20 and 50 μM compared to Veh ($p < 0.05$ and 0.01, respectively). The co-treatment of D3 with ICI inhibited the ERE-dependent luciferase activity. No statistical differences were observed between E_2-treated and D3+E_2-treated groups, suggesting that D3 did not exhibit antagonism or alter E_2-induced transcription.

D3 interacts with and translocates ERα to the nucleus. In the transfection studies, WT ERα is primarily located in the nucleus even in the absence of estrogen ligand (Burns et al. 2011). We then used H2 ERα as a tool to assess the ability of D3 to initiate direct ERα interaction and transactivation and translocation as a measure of D3-ERα interaction. Both E_2 (10 nM) and D3 (50 μM) significantly induced 3×ERE-Luc in the presence of H2 ERα (Figure 2B**;** $p < 0.001$ and 0.01, respectively). The transactivation activity induced by either E_2 or D3 is fully inhibited by ICI. The co-treatment of D3 with E_2 did not alter the transactivation induced by E_2.

Because the WT ERα is nuclear localized in the absence of ligand, we were unable to illustrate that D3 induced nuclear translocation using WT ERα. Therefore, we used H2 ERα-GFP transfected into HeLa cells to test D3 binding by visualizing that D3 increases the translocation of ERα to the nucleus. The D3 treatment caused increased H2 ERα-GFP signal in the nuclei similar to E_2 treatment (Figure 2C). To illustrate that the nuclear translocation induced by D3 is ERα-dependent, D3 was co-treated with ICI. ICI treatment alone induced a punctate pattern in the cytoplasm reminiscent of protein degradation, which is known to occur for ER with ICI treatment (Dauvois et al. 1993). Nuclear translocation of H2 ERα-GFP by D3 and E_2 treatment was disrupted by ICI co-treatment. Results indicate that D3 action was mediated through ERα

interaction. Nuclear and cytoplasmic H2 ERα-GFP intensities were quantitated and demonstrated that E_2 and D3 treatment resulted in a significant increase in the percentage of H2 ERα-GFP intensity in the nucleus when compared to Veh treated (Figure 2D, $p < 0.05$). ICI treatment, either alone or with ligands resulted in the higher percentage of cytoplasmic H2 ER-GFP intensity. Collectively, D3 induced ERα-interaction, translocation, and nuclear occupancy, thus D3 is able to mediate ERα activity.

D3 stimulates an ERα-dependent response in the uterus. Our results suggested that D3 utilized ERα and induced ERE-dependent transcription similar to E_2 *in vitro*. We then evaluated the transcriptional profile of D3 compared to E_2 in an *in vivo* uterine model using WT and αERKO animals. The physiological responses of the mouse uterus to E_2 consist of both early and late phase events (Hewitt et al. 2003). The effects of D3 on the early (2 h) and late (24 h) events were examined in OVX mice. D3 at a dose of 100 mg/kg was administered to the animals, as this dose previously exerted the maximal uterine responses (Winuthayanon et al. 2009a). At 2 h, E_2-regulated genes, *Fos* and *Inhbb*, were significantly up-regulated in WT mice treated with E_2 and D3 ($p < 0.01$ for *Fos*, and $p < 0.05$ for *Inhbb*) when compared to Veh (Figure 3A). *Aurkb* and *Ccnb2* were also significantly up-regulated in WT uteri after E_2 and D3 treatment at 24 h ($p < 0.01$) when compared to Veh (Figure 3B). Gene activation at both 2 and 24 h was not observed in either E_2 or D3-treated αERKO uteri, indicating the requirement of ERα for the early and late response activation by E_2 and D3.

D3 does not alter estrogen action in the uterus. We demonstrated the estrogenic action of D3 in the uterus was ERα-dependent; therefore, the following experiments were focused on the responses in WT animals. Uterine wet weight increase, epithelial cell proliferation, and PR expression patterns were evaluated as parameters of the late biological responses at 24 h. -

Biological responses were also assessed in the presence or absence of E_2 (10 μg/kg) to determine if D3 would exhibit anti-estrogenic activity in the uterus. As expected, E_2 treatment significantly increased uterine wet weight ($p < 0.05$); however, D3 (100 mg/kg) did not significantly stimulate uterine wet weight (Figure 4A). Co-treatment of D3 with E_2 neither augmented nor diminished the E_2-induced uterine wet weight increase. Although uterine weight was not significantly induced by D3 treatment, D3 did induce uterine DNA synthesis as shown by the positive signal of EdU incorporation in the uterine epithelial cells, similar to E_2 (Figure 4B). Together with E_2, D3 did not further alter the level of DNA synthesis in the uterine epithelium induced by E_2. To evaluate estrogen responsiveness, PR protein expression patterns were evaluated by immunohistochemical analysis. PR is expressed in the uterine luminal and glandular epithelium in the absence of ovarian hormones (after ovariectomy) as observed in Veh treated animal (Figure 4C). In the presence of E_2, PR expression decreases in the uterine epithelium, but increased in the uterine stroma (Tibbetts et al. 1998). Similar to E_2, D3 decreased PR expression in the uterine epithelium and increased PR in the stroma. Co-treatment of D3 with E_2 did not alter the expression pattern of PR induced by E_2, indicating that D3 has weak estrogenic agonist activity and does not exert anti-estrogenic effects on PR expression in the uterus.

Discussion

We previously showed that D3, a naturally occurring phytoestrogenic compound from *C. comosa*, exhibited estrogenic like activity *in vitro* and *in vivo* (Suksamrarn et al. 2008; Winuthayanon et al. 2009a; Winuthayanon et al. 2009b); however the underlying mechanism(s) of uterine action of D3 had not been investigated. The present study further characterized the mechanisms of the uterotrophic responses of D3 in human uterine cells as well as in an animal

model for comparison with an endogenous hormone, estrogen. Certain goals of this study were to extrapolate the implications of local use of this indigenous plant in women as a health promotion supplement and alternative treatment for post-menopausal symptoms; in addition to understanding more of the mechanism of action of this compound since it shows divergent estrogenic activity. Herein, we focused in detail on the transcriptional regulation mediated by ERα in human uterine cell lines and on the profile of different physiological events in uterine responsiveness during proliferation (early (2 h) and late (24 h) responses). We also explored the possible binding mode of D3 to the ligand binding domain of ERα via molecular modeling.

Historically compounds with agonist cores and large bulky side chains behave as antagonist to the ER by displacing helix 12 from the agonist binding position (Brzozowski et al. 1997). Thus, based on its structural properties it was surprising when Nettles et al. reported that TFMPV-E_2 could function as a potent agonist (Nettles et al. 2007). The crystal structure of TFMPV-E_2 bound to ERα revealed plasticity in the ERαLBD whereby the trifluoromethylphenylvinyl side chain can be accommodated by the unwinding and displacement of helix 7 and the rearrangement of a few side chains (Nettles et al. 2007). Binding in this manner increased the volume of the binding pocket by 40% while maintaining helix 12 in a position consistent with agonist binding. Interestingly, the structure of D3 can be reasonably superimposed onto the structure of TFMPV-E_2 bound to the ERαLBD in a remarkably similar conformation. The structure of D3 can be manipulated such that both phenyl groups superimpose with the phenol and phenyl groups of the TFMPV-E_2 compound. Binding in this orientation also positions the D3's hydroxyl oxygen in a similar location to that of 17β-hydroxyl of E_2. Although it is unclear if this is indeed the binding mode of D3 to the ERαLBD, it does support the possibility that this compound can bind in an orientation consistent with agonist binding and -

activity. In addition to our modeling, our previous finding using reporter assays in HepG2 cells indicated the AF2 domain within ERαLBD is crucial for D3 transcriptional activity, as mutation in AF2 domain blunted D3 mediated transcriptional responses (Winuthayanon et al. 2009b).

Estrogens exert their regulatory potential on gene expression in target tissues by different mechanisms. A number of compounds are able to interact with both ERα and ERβ (Kuiper et al. 1998). The uterus is one of the most estrogen-responsive reproductive tissues, which predominantly expresses ERα (Couse et al. 1997; Nilsson et al. 2001). The ligand-ER complex in the nucleus interacts with both ERE or non-ERE (tethered) sequences (Couse and Korach 1999). We previously reported that D3 transactivated genes through an ERα/ERE-dependent manner in human liver cells with no tethering activity (Winuthayanon et al. 2009a). The present study further investigated the mechanisms of action of D3 in uterine cells by introducing WT or H2 ERα in Ishikawa cells. In the uterine cells, D3 activated an ERα/ERE mediated luciferase reporter. However, to obtain a detectable biological response, D3 must be administered at a very high dose compared to E_2. Traditional $^3H[E_2]$ ligand binding assays using uterine cytosolic preparations were unable to demonstrate D3 binding to ERα (see Supplemental Material, Figure S1), which may due to the very low binding affinity of D3 to ERα as shown by the high dose required for both reporter gene activity and uterine bioassay, or may result from the use of the crude cytosolic preparation which contains binding proteins that may bind non-specifically to D3, preventing interaction with ERα. Therefore, we used the H2 ERα, a mutant that exhibits hormone-dependent translocation from the cytoplasm into the nucleus in the presence of ligand (Burns et al. 2011). We demonstrated that D3 treatment induced H2 ERα transactivation in both luciferase reporter assay, and H2 ERα-GFP translocation into the nucleus. Both findings suggest that D3 interacts directly with ERα. Herein, we also illustrated that H2 ERα could be a useful

and sensitive experimental tool in place of ligand binding assays of compounds that exerted weak estrogenic activity that could not be tested by the conventional ligand binding assay.

Endocrine disruptor compounds (EDCs), such as bisphenol A (BPA), and 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE), are considered 'weak estrogens', exhibiting early phase estrogenic responses in the uterus, but late responses are diminished after 24 h (Hewitt and Korach 2011). Similarly, we found that D3 stimulated the expression of early phase genes (*Fos* and *Inhbb*) in a fashion similar to E_2. D3, however in contrast to BPA or HPTE, also sustained its effect on the induction of late phase genes (*Aurkb* and *Ccnb2*). The transcriptional responses by D3 were mediated through ERα as shown by the lack of gene stimulation in αERKO uteri. In addition to the genomic responses, D3 clearly stimulated DNA synthesis selectively in uterine luminal and glandular epithelium, concomitant with the up-regulation of cell cycle related genes, such as *Aurkb* and *Ccnb2* (at 24 h). However, it is noted that the uterine weight did not significantly increase with D3 treatment at 24 h. One explanation for this discrepancy of the tissue response and gene activation is that a repetitive treatment is required for weak estrogens to induce the uterine weight increase as the gene expression changes may be more sensitive endpoint for stimulating the biological tissue response endpoints. In the previous studies, D3 significantly increased the uterine weight after administration for 2 and 3 consecutive days in immature OVX rats (Winuthayanon et al. 2009b) and adult OVX mice (Winuthayanon et al. 2009a), respectively. However, the significant uterine weight increase induced by D3 was still lower than that of E_2, which is consistent with the property of a weak estrogen. Thus, repeated dosing of D3 is required for the weight increase response; however, the uterine genomic responses of D3 can be observed at 2 and 24 h after a single injection. In addition, the gene expression pattern at 24 h was sustained by D3 treatment supporting its potential potency. We

also found that in the presence of an endogenous estrogen, 17β-estradiol, D3 did not alter the PR expression pattern induced by E_2. Note that the dose administered *in vivo* in the present study was 100 mg/kg (or 2.5 mg per mouse). From pharmacokinetic studies in rats, the bioavailability of D3 via oral administration is approximately 24.01% (Su et al. 2012). If mice with intraperitoneal administration had a similar bioavailability as rats, D3 at a dose of 100 mg/kg would have approximately a circulating level of D3 at 132.3 μM. This suggests that the dose of D3 used in the *in vivo* would be in a similar range to the dose used *in vitro*. In summary, D3 acts as a weak estrogen through ERα as shown *in vitro* and *in vivo* biological assays.

Conclusions

We demonstrate the biological actions of D3 are mediated by its transcriptional activity as an agonist for ERα through an ERE-dependent reporter in uterine cells and produces uterine responses in both the early and late phases in a manner similar to that of E_2 in the mouse uterus without interfering with the effect of endogenous estrogens. Surprisingly, we also showed that D3 had a unique chemical structure that could be accommodated in the binding pocket of ERα. Our 3D modeling may shed light on how other non-steroidal EDCs exert their estrogenic activity through ERα. D3 shows promise as a naturally isolated weak estrogenic compound that might be used as an alternative therapy for symptoms in women that result from estrogen withdrawal. However, D3 must be administered frequently at extremely high doses either *in vitro* or *in vivo* to produce maximal biological responses that approach but never equal E_2 responses. The identification and characterization of D3's actions on the molecular targets advance our basic knowledge of phytoestrogen D3's actions in the uterine cells in the presence of the endogenous hormone, E_2. This study also suggests that although it acts as a weak agonist D3 did not interfere

or antagonize the action of E_2 in the *in vivo* model, which may suggest the use of this plant in ovarian cycling women. Although diarylheptanoids are naturally occurring compounds abundant in spices and vegetables, the possibility of its proliferative DNA synthesis activity and increased risk for cancer should not be overlooked during long term consumption.

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Figure Legends

Figure 1. Potential binding mode of D3 to the ERα LBD. (*A*) Chemical structure of D3. (*B*) Superposition of the D3 model (yellow) onto the TFMPV-E_2 agonist (green) bound to the ERαLBD. (*C*) Superposition based on the crystal structures of E_2 bound to ERαLBD (magenta) and TFMPV-E_2 bound to the ERαLBD (green) reveals the conformational changes between the binding modes of the two agonists. Helix 7 unwinds in the TFMPV-E_2 binding mode and side chains M342, M421, and F425 all have altered conformations to accommodate the E_2 ligand substitution. D3 is superimposed and is shown in transparent yellow. Figure *B* and *C* were created using PyMol (DeLano 2012).

Figure 2. D3 transactivated and induced the shuttling of ERα from cytoplasm into the nucleus. D3 dose response curve of luciferase activity in Ishikawa cells in the presence of (*A*) mouse WT ERα and (*B*) H2 ERα mutant after 24 h treatment with Veh, 17β-estradiol (E_2 10 nM), ICI 182,780 (ICI 1 μM), ICI (1 μM) + E_2 (10 nM), D3 (1, 10, 20, 50 μM), ICI (1 μM) + D3 (50 μM), or D3 (50 μM) + E_2 (10 nM). Each value represents mean ± S.E.M. from the triplicates of three different experiments. *, **, and ***, $p < 0.05$, 0.01, and 0.001, significantly different from Veh. *ns*; not significant different. (*C*) H2 ERα-GFP expression in each cell compartment after treatment with Veh, E_2 (10 nM), D3 (50 μM), ICI (1 μM), ICI (1 μM) + E_2 (10 nM), or ICI (1 μM) + D3 (50 μM) for 3 h. The translocation of H2 ERα-GFP (green) from the cytoplasmic to nuclear compartment was visualized by confocal microscopy. DAPI (blue) was used to visualize the cell nucleus. All images represent the same magnification. Scale bar = 10 μm. (*D*) Quantification of H2 ERα-GFP colocalization. The signal from H2 ERα-GFP and DAPI were analyzed as percentage within each cell compartment compared to whole cell intensity (100-180

cells/group). Mean ± S.E.M. $^{\#}p < 0.05$, significantly different when compared with the nuclear GFP intensity of Veh treated sample.

Figure 3. D3 stimulates ERα-dependent early and late gene responses in the mouse uterus. (*A*) Early (2 h; *Fos* and *Inhbb*) and (*B*) late (24 h; *Aurkb* and *Ccnb2*) transcripts in the uterus after the treatment with Veh (sesame oil), E_2 (10 μg/kg), or D3 (100 mg/kg) in adult OVX WT and αERKO mice. Each value represents mean ± S.E.M. ($n = 4$). *, and **$p < 0.05$ and 0.01, significantly different from Veh within the genotype.

Figure 4. D3 induced the uterine DNA synthesis, but not uterine weight increase, in adult mice without an additive or antagonistic effects on E_2 treatment. (*A*) Normalized uterine weight of adult OVX females after treated for 24 h with Veh (sesame oil), E_2 (10 μg/kg), or D3 (100 mg/kg) with or without E_2 (10 μg/kg). Mean ± S.E.M. ($n = 3$-4). *ns*; not significant different from Veh. * and **$p < 0.05$ and 0.01, significantly different from Veh, respectively. (*B*) DNA synthesis in uterine luminal and glandular epithelium induced by D3. The fluorescent signal of EdU incorporation (S-phase of DNA synthesis) and Hoescht 33342 (DNA staining), and (*C*) Immunohistochemistry (IHC) of PR protein expression patterns in the uterine sections. Representative images shown and represent the same magnification. Scale bar = 100 μm.

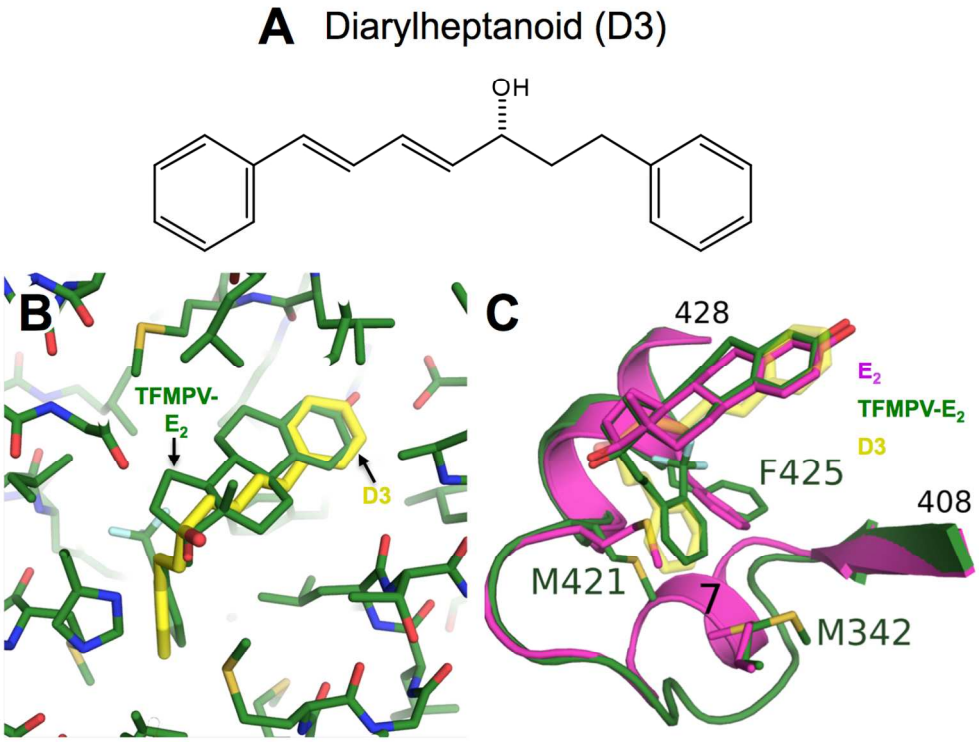

Figure 1
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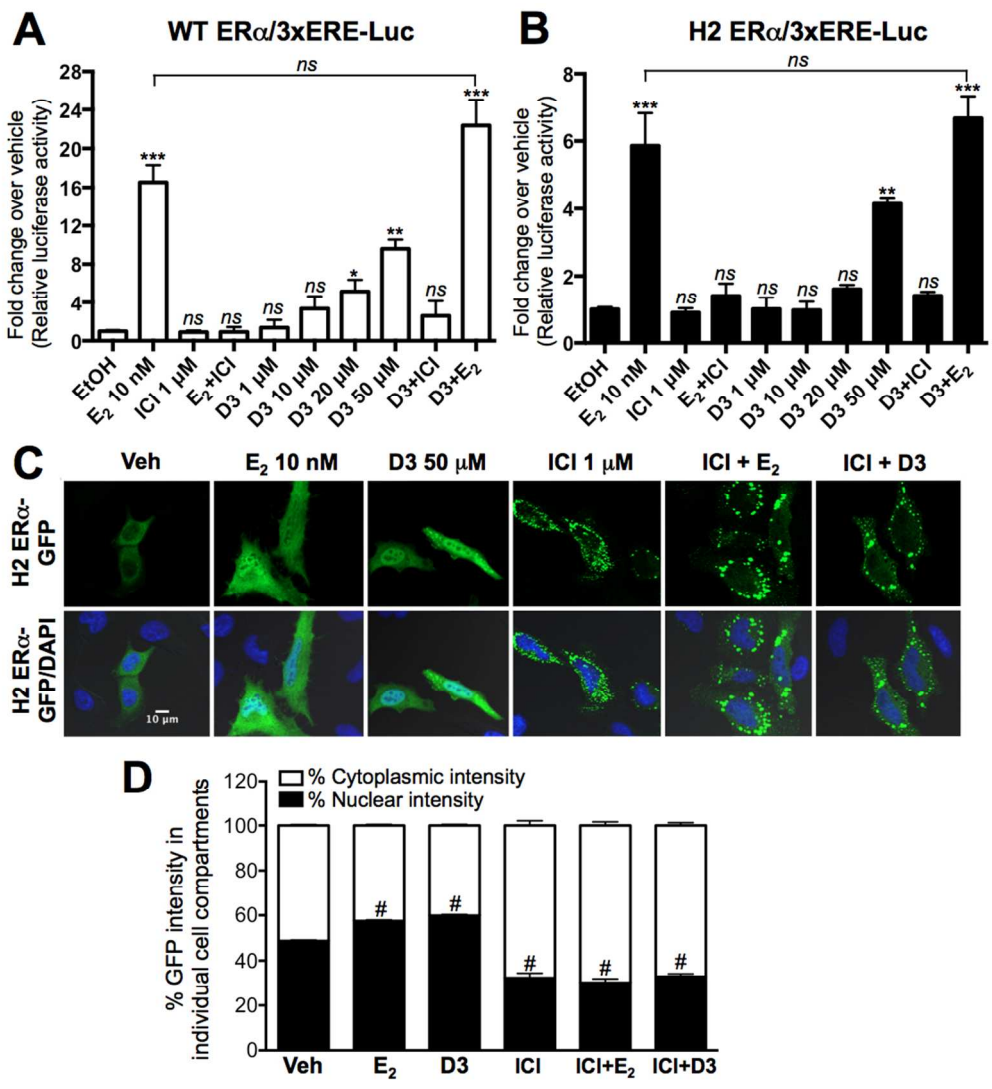

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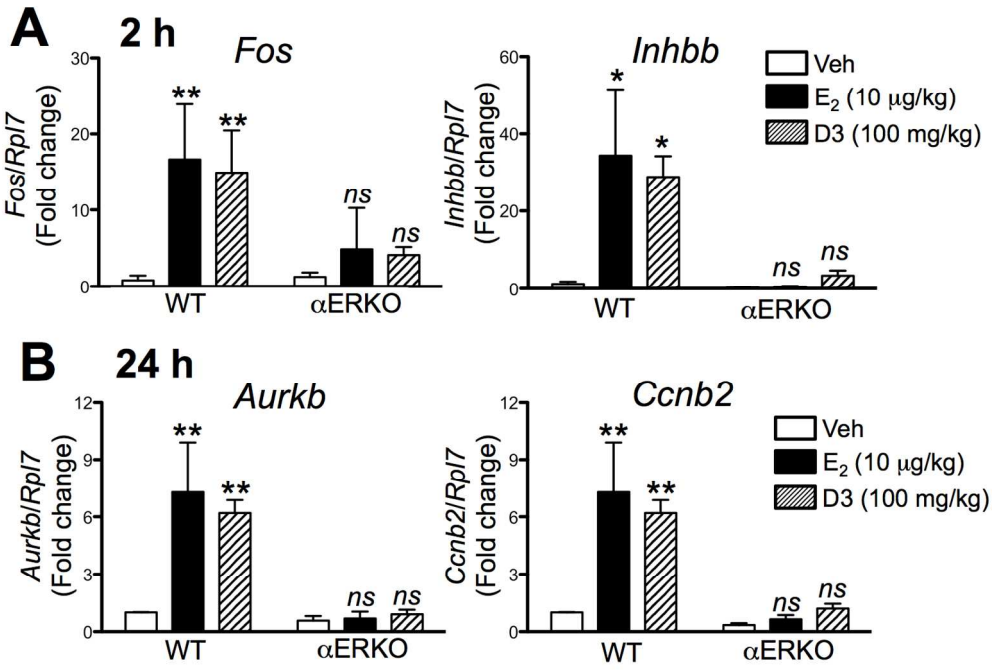

Figure 3
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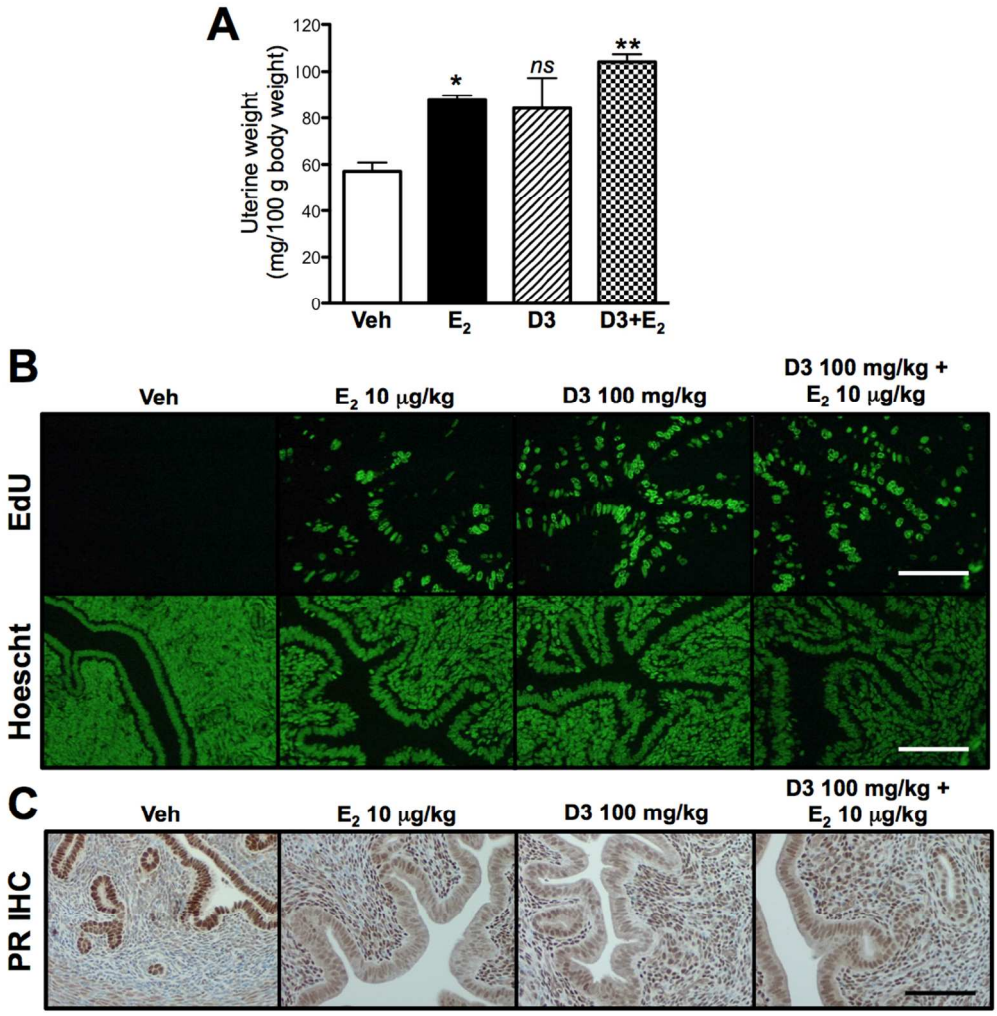

Figure 4
415x424mm (72 x 72 DPI)